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Issue: *Thymosins in Health and Disease***Progression of ocular sulfur mustard injury: development of a model system**Denise Milhorn,¹ Tracey Hamilton,² Marian Nelson,² and Patrick McNutt²¹United States Army Medical Research and Materiel Command, Fort Detrick, Maryland, USA. ²United States Army Medical Research Institute of Chemical Defense, APG, Maryland, USA

Address for correspondence: Patrick McNutt, PhD, Research Division, USAMRICD, 3100 Ricketts Point Road, APG, MD 21010, USA. patrick.mcnu@us.army.mil

Exposure of tissues to sulfur mustard (SM) results in the formation of protein and nucleotide adducts that disrupt cellular metabolism and cause cell death. Subsequent pathologies involve a significant proinflammatory response, disrupted healing, and long-term defects in tissue architecture. Following ocular exposure, acute corneal sequelae include epithelial erosions, necrosis, and corneal inflammation. Longer term, a progressive injury becomes distributed throughout the anterior chamber, which ultimately causes a profound remodeling of corneal tissues. In many cases, debilitating and vision-threatening injuries reoccur months to years after the initial exposure. Preliminary data in humans suffering from chronic epithelial lesions suggest that thymosin $\beta 4$ (T $\beta 4$) may be a viable candidate to mitigate acute or long-term ocular SM injury. To evaluate therapeutic candidates, we have developed a rabbit ocular exposure model system. In this paper, we report molecular, histological, ultrastructural, and clinical consequences of rabbit ocular SM injury, which can be used to assess T $\beta 4$ efficacy, including timepoints at which T $\beta 4$ will be assessed for therapeutic utility.

Keywords: thymosin- $\beta 4$; sulfur mustard; rabbit ocular injury

Introduction

Sulfur mustard (2,2'-dichloroethylsulfide; SM) is a highly reactive alkylating chemical that causes debilitating clinical morbidities following topical or inhalational exposures. Chemical and physical properties such as environmental persistence and ease of synthesis and delivery make it a particularly effective chemical warfare agent. The use of SM in WWI, WWII, and the Iran-Iraq war resulted in pulmonary, ocular, and dermal injuries in over 50,000 casualties.¹ Eyes are roughly 10-fold more sensitive to SM injury than skin, largely due to the aqueous-mucous interface and high metabolic rate of corneal epithelial cells. Therefore, it is not surprising that approximately 75% of SM casualties report ocular injury, with 10% presenting with severe ocular damage.² In both humans and experimental animals, the acute stage of injury lasts 1–2 weeks, and includes conjunctivitis, photophobia, and corneal swelling. Following a latent period characterized by mini-

mal clinical evidence of corneal injury, both human and experimental animals undergo a delayed, more severe injury that can result in irreversible visual impairments and even blindness.³

Initial symptoms of mustard gas exposure are clinically evident within 1 h, and include a feeling of grittiness, soreness, and corneal injection. At 2–6 h, the corneal epithelium (CE) begins to slough, leading to severe pain, photophobia, corneal edema, and impaired vision. Reepithelialization of the acute lesion is usually underway by 2 days after exposure, with restoration of a basal epithelial cell layer by 4–5 days. Victims of a mild exposure are typically asymptomatic within weeks, although more severe exposures can result in prolonged conjunctivitis, corneal opacity, or even corneal ulceration and impaired vision.^{4–7} A form of delayed ulcerative keratopathy often develops in an estimated one-quarter to one-half of exposed patients, with reported latencies between 1–40 years. The pathogenic mechanisms of this condition are still unknown, but the most

common clinical sequelae include chronic epithelial lesions, corneal neovascularization, a progressive corneal ulceration and frequent impairment or loss of vision.^{8–10} Similar phenomena have been reported in whole eye exposures in rabbits, with latent periods comprising weeks rather than years.¹¹

To date there is no definitive treatment for the delayed keratitis caused by SM. Current therapeutic strategies provide a microenvironment conducive to healing, such as through artificial tears, bandage contact lenses and immunomodulatory drugs, but otherwise depend on the eye's innate ability to self-repair.^{12–14} Although much progress has been made in understanding how the cornea responds to acute insults, the design of SM treatment strategies is complicated by the lack of knowledge regarding the etiology of recurrent corneal dysfunctions. Since symptoms of this delayed keratitis are distributed throughout the anterior chamber, we believe an effective therapeutic strategy is likely to require a combination of drugs and delivery method(s) that ensures treatment of multiple corneal tissues. Thymosin β 4 (T β 4) is a highly conserved, 43 amino-acid polypeptide that is constitutively expressed in a variety of tissues and cells and has been shown to influence cell migration, proliferation, and differentiation when applied ectopically.^{15,16} In a mouse model of alkali injury, T β 4 administration promotes corneal wound healing, decreases inflammation, and modulates activity of matrix metalloproteases.¹⁷ In a compassionate human use study, ectopic T β 4 delivery comprehensively resolved chronic epithelial erosions related to diabetes mellitus.¹⁸ These studies suggest T β 4 may provide therapeutic utility as one piece of a multifactorial antidote for SM injuries.

We have developed an ocular vapor SM exposure model and established clinical and histopathological metrics of injury and healing. Here we present our initial characterization of this model system, elaborate on specific aspects of injury progression that may be responsive to T β 4 treatment, and identify metrics with which to evaluate the therapeutic consequences of T β 4 administration.

Results and discussion

Overview of the rabbit exposure model

We have developed a rabbit SM vapor exposure model to characterize ocular injury progression

and evaluate therapeutic interventions. Rabbits have several anatomical and physiological features that are experimentally advantageous for this effort. The rabbit cornea is structurally similar to the human, and although rabbit are fourfold less sensitive to ocular SM injury, at functionally equivalent doses rabbit and human eyes exhibit nearly identical lesions.^{6,19} Rabbits have a large ratio of cornea to sclera, which facilitates the ability to incur and study corneal injury. Finally, rabbits rarely experience spontaneous epithelial lesions, and a delayed blink reflex and relative insensitivity to corneal drying means there is little risk of complications during ocular exposures and clinical evaluations.

Morphologically, the rabbit cornea consists of a four- to six-cell-thick stratified epithelium that rests on a basement membrane. More permanent anchorage is provided by hemidesmosomal attachments to a specialized region in the anterior stroma called Bowman's layer (BL). BL transitions to the highly organized stroma, which is predominantly composed of collagen with distributed fibrocytes. Beneath the stroma is Descemet's membrane, which serves as the basement membrane for a single layer of endothelial cells that act as a barrier between the corneal tissue and the aqueous humor (see, for reference, Fig. 4A–C). The epithelial and endothelial cell layers actively maintain the stroma in a largely dehydrated state, which is essential for proper corneal function. Of these tissues, the BL cannot be repaired and the stroma and endothelium have very limited ability to self-repair.

Exposures and metrics to evaluate injury progression

Characterization of corneal-specific aspects of SM injury progression is complicated by direct injury to noncorneal ocular tissues, such as the eyelids, sclera, conjunctiva, and limbus. To specifically target SM delivery to the cornea, we used a novel vapor cup delivery system applied directly over the central cornea. Anesthetized New Zealand White rabbits were exposed for 1–4 min, and the contralateral eye served as a control. Animals were evaluated at regular intervals using clinical metrics such as fluorescein staining to measure epithelial lesions; pachymetry and slit lamp examination to determine corneal thickness; and gross evaluation to assess ocular morphology and injection. Histopathological metrics included ultrastructural analysis by

transmission electron microscopy (TEM) and microscopic assessment of hematoxylin and eosin-stained sections. In select cohorts, biochemical analysis of proinflammatory mediators was conducted in aqueous humor collected by longitudinal paracentesis.

Initial evaluation of reproducibility and injury progression

We evaluated the reproducibility of vapor delivery by quantifying the plasma concentrations of SM:protein adduct at 24 h. We found that the vascular accumulation of plasma adducts strongly correlates with duration of exposure between 1 and 4 min of vapor delivery (Supporting Fig. S1; $R^2 > 0.98$). Because the stroma is predominantly avascular, the mechanism by which SM:protein adducts accumulate in the vascular system is currently unknown. Possible explanations could include penetration to the aqueous humor, lateral movement of the SM before it is hydrolyzed (SM has a half-life of 8 min in aqueous media), and uptake into the superficial vasculature.^{20,21}

The resulting injury was clinically evaluated over a 9-week period in 24 animals, allowing us to evaluate whether injury progression was influenced by the duration of exposure. The presentation of acute injury is largely dose dependent; longer exposures resulted in increased and more persistent corneal opacity, injection, and stromal edema. As described later, the delayed injury was significantly more severe than the acute, and indistinguishable by histopathology among the different doses. Based on the severity of the initial injury and the frequency of

occurrence of the delayed injury, we chose to use a 2.5 min exposure. This dose generates a reasonably well-tolerated acute injury that appears clinically resolved within 1–2 weeks, yet results in a high frequency of recurrent injury starting between 3 and 5 weeks.

Characterization of acute injury

To characterize the acute injury, 28 animals were exposed to vapor SM for 2.5 min. Acute ocular injury progression was evaluated on a daily basis for the first week using clinical assessments, histopathology, ultrastructural analysis, and standard chemistry panels. Blood chemistry panels showed no significant differences between injured and control animals (data not shown). Clinical evaluations with fluorescein staining demonstrate gross epithelial lesions with sharply defined boundaries 24 h after exposure (Fig. 1). As of 48 h, lesion margins exhibited variegations, suggesting either immigration of CE cells or an expansion of the erosion. Changes in the size and shape of the margins between 24 and 48 h suggest a dynamic interplay between expansion and immigration. The lesion is either significantly reduced in size or gone by 72 h, and is completely resolved in all animals by 96 h ($n = 8$). Planimetric analysis suggests CE reepithelialization rates exceed $0.15 \text{ mm}^2/\text{h}$, consistent with reports of $0.12\text{--}0.27 \text{ mm}^2/\text{h}$ after wounding by scrape injury or chemical burns.²²

Pachymetry readings (Fig. 2, top right panel) demonstrate a significant increase in corneal thickness from $\sim 300 \text{ }\mu\text{m}$ at baseline to $\sim 1500 \text{ }\mu\text{m}$ at the peak of inflammation (day 4). Corneal thickness is

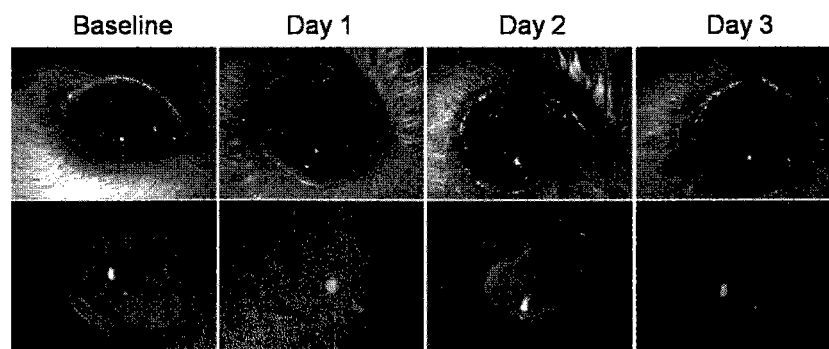


Figure 1. Longitudinal panel comparing white light and fluorescein staining in a representative rabbit eye following ocular vapor SM exposure for 2.5 min. From left to right are baseline (preexposure) and days 1–3. White light pictures were imaged at $6.3\times$, fluorescein images were taken at $10\times$.

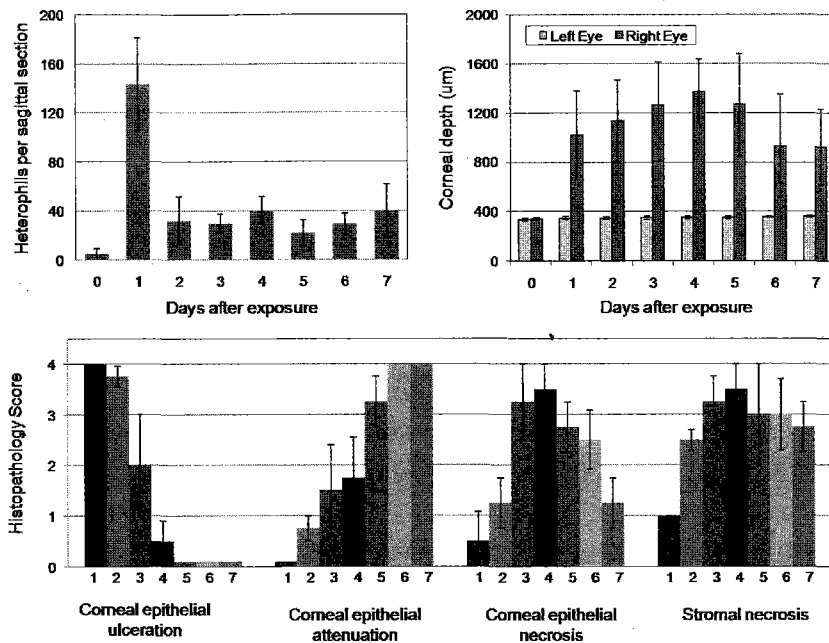


Figure 2. Longitudinal measurements of corneal thickness, neutrophil infiltration, and histopathological evaluations over 7 days following a 2.5 min ocular vapor SM exposure. *Top left panel:* Longitudinal characterization of neutrophil recruitment into corneal tissue ($n = 8$). All data points are significantly different from controls ($P < 0.05$) and days 2–7 are significantly different from day 1 ($P < 0.05$). *Top right panel:* Pachymetry readings comparing the corneal thicknesses of left eye (naïve) and right eye (exposed; $n = 8$). “Baseline” represents readings made 1 day prior to exposure. Note there is no difference between baseline and left eye data any time point, suggesting there are no contralateral effects during the first week following SM exposure. *Bottom panel:* Summary of histopathology data for animals euthanized daily over the first week following a 2.5 min ocular SM vapor exposure ($n = 4$ at each day). For all figures: “0” represents no evidence of injury; “1” represents minimal evidence (injury is present over $<10\%$ of the area); “2” represents mild injury (11–25%); “3” represents moderate injury (26–45%); and “4” represents severe injury (46–100%).

reduced to 64% of the peak average value by day 6. In separate experiments, we found that corneal thickness remains constant from day 6 through at least 3 weeks ($P < 0.05$). Acute edema is likely caused by penetration of tears and inflammatory cells into the stromal tissue through epithelial erosions. This interpretation is consistent with the observation that the corneal thickness begins to decrease following reepithelialization. Interestingly, although the corneal thickness is still elevated threefold above baseline levels 1 week after exposure, white light evaluations suggest minimal injection and only a mild amount of ocular haze. Slit lamp analysis suggests much of the edema is localized to the stroma (data not shown).

Histopathological analysis supports clinical evidence of a highly dynamic healing response over the

first 7 days after exposure (Fig. 2, bottom panel). Consistent with the fluorescein data, SM-induced corneal lesions are present in all animals 1 day after exposure. Reepithelialization by immigrating CE cells is evident as soon as day 2 and complete by day 5 in all animals. Concomitantly, attenuation in peripheral CE is evident by day 3 as wing cells migrate from marginal tissue onto the newly epithelialized surface. Although stromal neovascularization was not detected within the first week, we did observe moderate edema and mild stromal deformity. The presence of neutrophils (aka, heterophils) migrating from epithelial vasculature into the stroma is increased 20-fold at 24 h (Fig. 2, top left panel; $P < 0.005$), followed by a rapid decrement to a persistent level of neutrophil infiltrate that is approximately fivefold higher than baseline

($P < 0.05$). There is also significant stromal fibrocytosis by 24 h.

TEM images of naïve (Fig. 3A) and exposed corneas (Fig. 3B–3D) corroborate with clinical and histopathological data. Control corneas have hemidesmosomal attachments, characteristic desmosomal adhesions, and a well-articulated epithelial–stromal architecture (Fig. 3A). As of 7 days postexposure, epithelial lesions have reepithelialized and are undergoing expansion and stratification; however, they still lack well-developed hemidesmosomes (Fig. 3B and 3D). The CE also exhibits an infiltration of white blood cells, significant edema, and the accumulation of protein in extracellular spaces, which complicates reestablish-

ment of hemidesmosomal attachments. Remnants of cytosol fibrocytes and neutrophils are abundant in Bowman's layer and the anterior stroma (Fig. 3C and 3D).

Evidence of a longer term, recurrent injury

In humans, ocular exposure to SM results in an acute injury followed by a recurrent injury after a latent period of months to years. To evaluate whether the rabbit exposure model undergoes a similar progression, we evaluated exposed animals over 9 or 16 weeks following SM exposure. Aqueous humor was collected by paracentesis on a bimonthly basis and ocular tissues were examined by light microscopy and TEM after necropsy.

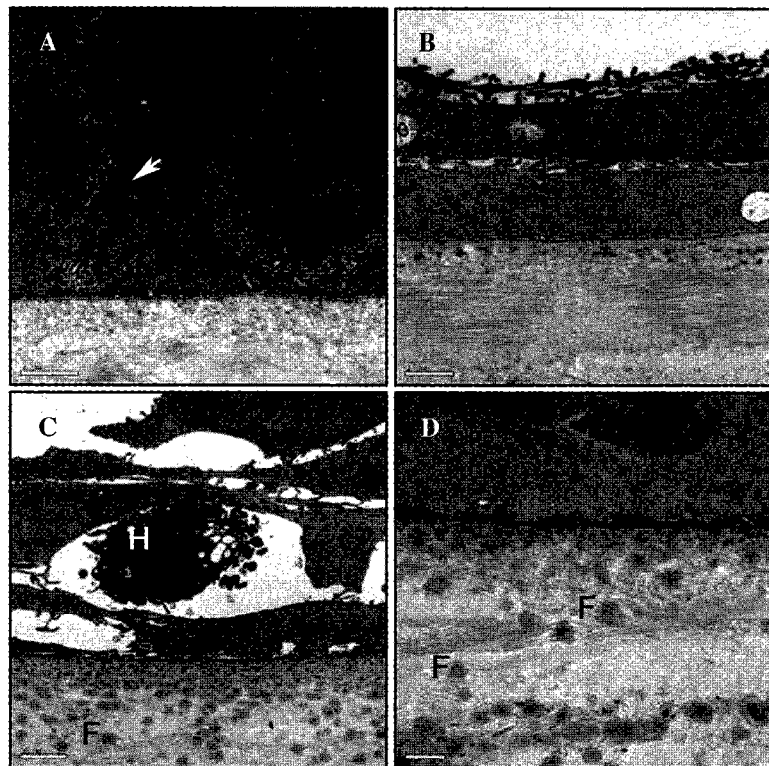


Figure 3. Transmission electron microscopy of corneas 7-days post SM-exposure. (Panel A) Image of naïve cornea showing hemidesmosomal attachments (*black arrowhead*), desmosomal attachments (*white arrow*), and a well-articulated epithelial–stromal boundary. (Panel B) One week after exposure, a basal epithelial cell layer has been reestablished, although hemidesmosomal junctions are not well developed (see inset panel D). A second cell layer has been generated and epithelial stratification is underway. (Panel C) SM induces epithelial edema and attenuation, infiltration of neutrophils (H), loss of hemidesmosomes, and mineralization of dead fibrocytes (F) within the stroma. (Panel D) Inset from panel B showing lack of organized hemidesmosomal attachments and fibrocytic mineralizations (F) in the anterior stroma.

Consistent with reports in both human victims and experimental animals, SM exposure induced a delayed injury following a 1- to 3-week period of apparent clinical latency.⁸⁻¹¹ We found that 33% of animals that received less than 2 min of vapor SM experienced a recurrent injury ($n = 6$), as opposed to 84% of animals that received between 2 and 4 min ($n = 19$). Interestingly, although the probability of a recurrent injury was higher with longer exposures, by histopathological assessments, there was no detectable difference in the severity of recurrent injuries. We are currently investigating this finding in greater detail, because it may provide significant insight into pathogenesis of the recurrent condition.

We initially characterized longitudinal changes in injury progression over a 9-week period following a 2.5 min exposure by white light photography on a weekly basis (Supporting Fig. S2). One week after exposure, eyes typically exhibit a mild corneal

haze that resolves during the following 2 weeks, depending on the length of exposure. Between weeks 3 and 5, there is an increased degree of corneal haze and vascular injection, with concomitant photophobia and obvious discomfort. Gross epithelial lesions spontaneously occur and rapidly reepithelialize. Following euthenasia at week 9, ultrastructural examination demonstrates a significant disruption of the basal lamina, loss of hemidesmosomes and epithelial attachments, infiltration of macrophages, and accumulation of cell debris in the epithelial layers (Supporting Fig. S3). Barrier dysfunction causes edema throughout the epithelial layers. This injury is far more extensive than that seen after 1 week, and unlike the acute injury, is detectably distributed throughout the anterior chamber.

Evaluation of exposed animals 16 weeks after a 4 min exposure demonstrates a profound and widely distributed corneal disruption (Fig. 4). Uninjured corneas have cleanly organized stromal layers with

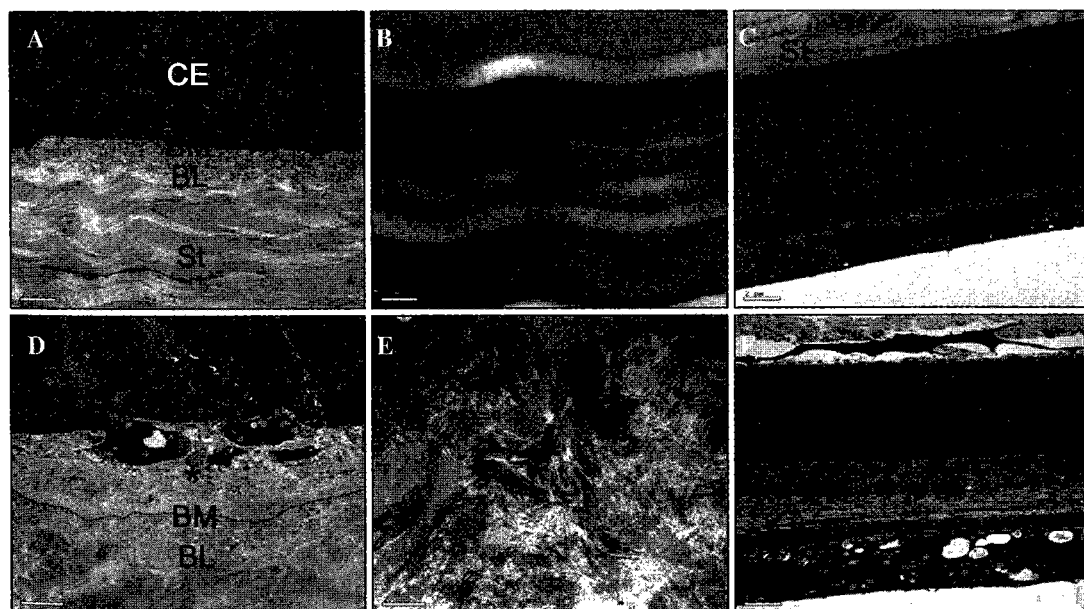


Figure 4. Transmission electron microscopy of rabbit cornea 16 weeks after a 4 min SM exposure. (A, B, C) Uninjured tissue. (D, E, F) SM-exposed tissue. (A, D) SM causes a chronic separation of the basal corneal epithelial cells from the basement membrane at Bowman's layer and disrupts hemidesmosomal attachments to the anterior stroma. The resulting edema (*asterisk*) contains cellular debris and infiltrating macrophages. Note the well-organized corneal epithelium (CE), basement membrane (BM), Bowman's layer (BL), and stroma in A vs. D. (B, E) Significant stromal remodeling and edema (*asterisk*) is profound in this animal 16 weeks after exposure. Note the lack of a laminar organization, lack of fibrocytes, and gross architectural disruption. (C, F) Severe edema (*asterisk*) and disorganization in the posterior stroma, Descemet's membrane (DM), and endothelium (Endo) that clearly demonstrates significant barrier dysfunction.

resident fibrocytes, stratified CE with characteristic desmosomal and hemidesmosomal junctions, and clearly defined Descemet's membrane and endothelium. Conversely, injured eyes are clearly undergoing profound corneal pathology. Stromal collagen is disorganized, edemic, and afibrocytic. There is significant edema and acellular protein between the basal epithelial cells and the basement membrane preventing the organized reestablishment of hemidesmosomal junctions. Edema is evident within the epithelial layer, and desmosomal attachments are sparse. The deeper cornea is also severely edemic and the endothelium, Descemet's membrane, and stroma interfaces are highly disorganized. We have not yet compared TEM images from different doses at 16 weeks, so cannot state whether a shorter exposure results in a similarly profound corneal remodeling.

Evidence of a delayed proinflammatory response

To assess whether exposure affects the composition of aqueous humor (AH), AH was longitudinally collected and analyzed for inflammatory cytokines, chemokines, and matrix metalloproteinases (MMPs) in eight animals over a 16 week period following a 2.5 min exposure. We observed a significant increase in interleukin (IL)-1 β , tumor necrosis factor- α , IL-6, and IL-8 with a peak at 7 weeks (data not shown). Zymographic analysis of aqueous humor demonstrated an increase in MMP-2 and -9 activities with a peak at 9 weeks (data not shown). Analyses of inflammatory mediators over time indicate that the SM injury progresses to the posterior chamber of the cornea, initiating the production of cytokines/chemokines, which in turn may activate MMPs. MMP-2, and -9 degrade collagen, and may contribute to the deterioration of the stroma. The temporal expression of these mediators suggests they contribute to the remodeling of the corneal structure as seen in Figure 4D–4F.

Summary and therapeutic approaches

We present evidence that SM-induced injury is distributed throughout the anterior chamber of the cornea, and involves direct and indirect damage to the epithelium, stroma, and the endothelium. Although the acute injury is well characterized, the underlying causes of long-term corneal pathologies are still unknown. The healing process of the

CE is dynamic and usually initiates within hours of injury. Architectural disruption of epithelial adhesion in SM treated cornea—for example, by destroying the corneal basement membrane or disorganizing the anterior stroma to the extent that reformation of hemidesmosomes is inhibited—may provide a partial explanation for long-term pathologies such as recurrent epithelial erosions. Notably, the ability of T β 4 to resolve chronic epithelial ulcers suggest that topical application may support the appropriate reestablishment of epithelial attachments.^{16,18,23,24}

Following a brief refractory period characterized by minimal clinical evidence of ocular injury, a large fraction of exposed eyes undergo a significant, recurrent, progressive injury that starts around weeks 3–5. Interestingly, the frequency of reoccurrence of injury is correlated to the degree of exposure (minutes), whereas the severity of the recurrent injury is significantly worse than the initial injury among all animals. Thus, this injury can be roughly divided into three phases; the acute injury (1–2 weeks), followed by a clinically quiescent “bridge” period, and recurrence of the injury weeks later (summarized in Supporting Fig. S4).

T β 4 has been demonstrated to promote corneal reepithelialization and epithelial cell migration, modulate polymorphonuclear cell infiltration, and inflammatory mediators, and inhibit apoptosis.^{17,25,26} We interpret these findings to suggest T β 4 may contribute to a combination therapy to mitigate the effects of SM-induced ocular injury. However, due to the plethora of mediators contributing to the onset of the acute and recurrent injuries, identifying a therapeutic point of application is challenging. We are evaluating several timepoints to determine if application of T β 4 by itself can mitigate the delayed injury (Supporting Fig. S4): (a) during the first week following exposure, (b) during the “bridge” period 2–3 weeks postexposure, prior to the manifestation of the recurrent injury, (c) at the onset of the recurrent injury at weeks 4–5, (d) at the peak of AH inflammatory cytokine/chemokine and MMP production at 7–8 weeks post exposure, and (e) at late stage injury to relieve recurring epithelial erosions. Other components of a multifactorial treatment are likely to include nonsteroidal anti-inflammatory drugs and a pharmacologic to support endothelium function or regeneration.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Quantification of SM-adduct concentration in plasma 24 h after ocular vapor exposure.

Figure S2. Longitudinal panel demonstrating gross injury progression over an 8-week period following 2.5 min ocular vapor SM exposure.

Figure S3. Transmission electron microscopy of corneas 9 weeks after a 2.5 min SM exposure.

Figure S4. Key aspects of SM vapor-induced ocular injury.

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Conflicts of interest

The authors declare no conflicts of interest.

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